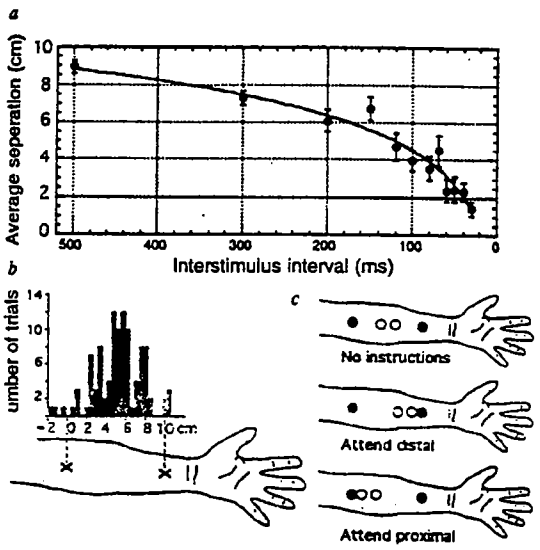
EX. F

Anticipated stimuli across skin

— Our perceptions occur only after the mation we receive from our senses has ...n heavily preprocessed. The degree to which cognitive influences shape this processing has been debated for many years. Sensory saltation is a robust class of illusions that has been described as a shift in the perceived location of a stimulus towards a rapidly delivered subsequent stimulus[1,2]. Our studies show that stimuli evoked in < 200-ms epochs are all mislocalized in sensory saltation. In the simplest two-stimulus form of the illusion, perceived stimulus locations are symmetrically shifted towards one another. A misunderstanding of the fundamental nature of the illusion apparently arose because powerful effects of directed attention on absolute stimulus localization were not recognized.



a, Perceived stimulus separation as a function of ISI. The forearm was hidden from the view of 13 naive subjects (Ss). After 10 repetitions of each ISI, Ss marked the locations of the two experimental taps with respect to the two locator taps. Bars are standard errors; $R = 0.97$. *b*, Distribution of centre points of experimental tap pairs, with data pooled for 30-, 40-, 50- and 60-ms ISIs. Mean location: 5.26 cm, s.d., 2.41 cm. *c*, Representative examples of the perception of the two locator and the two experimental stimuli for an ISI of 60 ms without (top) or with specific instructions that influenced their directed attention. Filled circles, actual stimulus locations; open circles, perceived stimulus locations.

Geldard and colleagues discovered sensory saltation and described versions of it in the tactile, auditory and visual systems studying its tactile form as their principal model[2]. In their most basic tactile paradigm, a skin tap that served as a reference (the 'locator') was delivered 500 ms before the presentation of two experimental taps ...h that it did not influence the mislocal... on. The first experimental tap was ...vered at the locator site; the second 10 cm up or down the forearm. Geldard's subjects perceived the first experimental tap to be nearer to the second (the 'attractor', which was properly localized) as a linear function of interstimulus interval (ISI), for ISIs below about 200 ms. For example, with an ISI of 100 ms, the second tap was perceived to be halfway between the actual loci of the two experimental taps, that is, mislocated by 5 cm.

A long-debated problem with Geldard's explanation of sensory saltation concerns how a brain mechanism can make a decision about where to locate a stimulus if the following 'attractor' has not yet occurred[3,4]. Our findings demonstrate that this apparent paradox arose from a misunderstanding of the illusion. Geldard and colleagues used trained subjects to reduce response variability. By training them, an important aspect of the illusion, a powerful biasing effect of directed attention (or expectation), was apparently circumvented.

We added a fourth stimulus at the second stimulus site as a second 'locator,' again well separated in time from the two experimental taps. In this simple symmetrical variation of Geldard's paradigm, naive subjects had no difficulty estimating the perceived separations between the two rapidly successive stimuli. However, although estimation of the absolute locations of the two stimuli on the forearm was quite variable, saltation stimuli were not perceived by naive subjects as one tap mislocalized towards the second, but rather, as taps located progressively closer to one another as the ISI was decreased (*a* in the figure). The centre points between the two perceived taps were distributed as a roughly gaussian function centred at a point midway between the two actual stimulus locations (*b* in the figure).

A main source of the substantial variability in locating the two events on the skin was made apparent when naive subjects were asked to concentrate on the proximal (or distal) region of the forearm. When so instructed, perceived separations between the two taps did not change, but the perceived skin locations of the two taps were dramatically shifted up or down the forearm in the attended direction (*c* in the figure). The average difference between the centres of the perceived locations when naive subjects were instructed to attend to the proximal versus the distal forearm was 3.1 ± 0.47 cm (mean ± s.e., $P<0.0001$). With practice, this powerful effect progressively strengthened, and could result in stimulus mislocalization exactly as Geldard described — or in an equivalently great opposite-direction mislocalization.

Thus, although the perceived separations of stimuli were determined by their physical separations and ISIs, the perceived locations of the stimulus pairs were largely determined by a subject's selective attention and/or expectation. We conclude that, in training, Geldard's subjects were effectively taught to concentrate on the 'attractor' location to reduce response variability.

A better understanding of the cutaneous saltation illusion gives us further insight into the fundamentals of tactile and haptic perception. The mechanisms that generate cutaneous saltation (and presumably related phenomena in vision and audition) operate as a smoothing process by which the perceived distances between two events occurring within a short time domain are generated by symmetric convergence, with locations powerfully biased by ongoing cognitive estimates of where those events are most likely to have occurred.

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Improved green fluorescence

SIR — The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has attracted widespread interest since the demonstration[1] that heterologous expression of the cloned gene[2] can generate striking green fluorescence. Despite the tremendous potential of recombinant GFP as a marker for gene expression or cell lineage or as an *in situ* tag for fusion proteins[3], the wild-type protein from *A. victoria* has several significant deficiencies. Its excitation spectrum (*a* in the figure) shows peaks at both 396 and 475 nm. The longer-wavelength excitation peak has the advantages of greater photostability[1] and better matching to standard fluorescein filter sets, but is relatively low in amplitude. Considerable improvement should be possible, because a closely related protein[4] from the sea pen *Renilla reniformis* has the same high quantum yield of emission (0.7–0.8), yet shows only one absorbance and excitation peak with an extinction coefficient per monomer more than 10 times that of the longer-

wavelength peak of *Aequorea* GFP[5,6]. We now report that simple point mutations in *Aequorea* GFP ameliorate its main problems and bring its spectra much closer to that of *Renilla*.

Serine 65 of the amino-acid sequence of *Aequorea* GFP becomes part of the *p*-hydroxybenzylideneimidazolinone chromophore. To test the hypothesis[7] that Ser 65 undergoes additional dehydration to form a vinyl side chain, we mutated that residue to Ala, Leu, Cys or Thr. If a vinyl group were formed by elimination of H_2O or H_2S, Ser and Cys should give identical spectra very different from Ala and Leu in which elimination is impossible. Serendipitously, all four mutants showed single excitation peaks, located at 470–490 nm, whose amplitudes were four- to sixfold greater than that of wild-type for equal numbers of molecules (*a* in the figure). These results exclude vinyl formation. The Ser 65→Thr mutant (S65T) was selected for further characterization because it had the longest wavelengths of excitation and emission (490 and 510 nm), which closely resembled those reported for *Renilla* GFP (498 and 508 nm). The crucial post-translational oxidation[8] to produce the fluorophore from the nascent polypeptide chain proceeded about fourfold more rapidly in S65T than in the wild-type protein (*b* in the figure). This acceleration ameliorates a poten-tially significant limitation in using GFP as a reporter protein for rapid gene inductions[9].

Mutations of Ser 65 to Arg, Asn, Asp, Phe, and Trp gave fluorescence intensities well below that of wild type. It remains unclear exactly how position 65 controls spectral properties or why *Aequorea* chose serine. Nevertheless, the greatly increased brightness and rate of fluorophore generation in mutants such as S65T should make them superior to wild-type *Aequorea* GFP for most experimental uses.

Note added in proof: GFP variants generated by combinatorial mutagenesis of positions 64–69 have excitation peaks near 490 nm, but their amplitudes and the kinetics of fluorophore formation have not been quantified[12].

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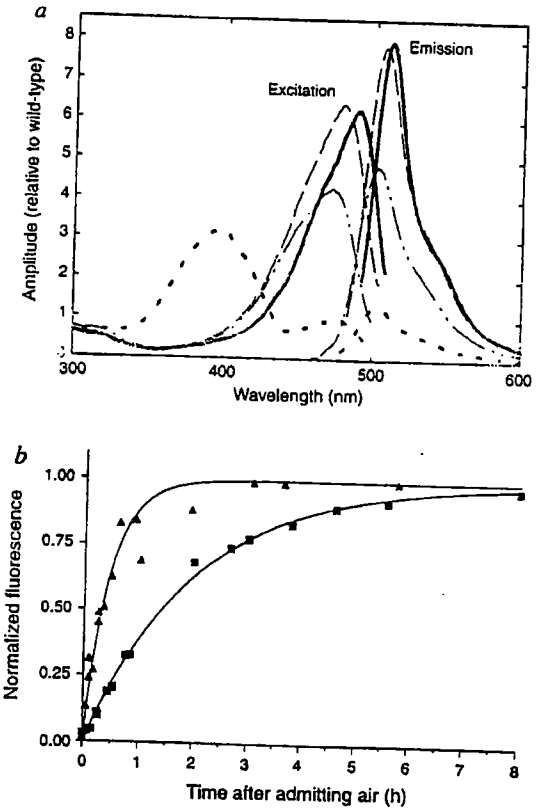

Comparison of recombinant wild-type and mutant green fluorescent proteins. *a*, Fluorescence excitation and emission spectra of wild-type protein (– – –), Ser 65→Ala (– · · –), Ser 65→Cys (– –) and Ser 65→Thr (—) mutants. The coding region of *Aequorea gfp* cDNA[2] (gift of D. Prasher) was cloned into the T7 expression vector pRSET$_B$ (Invitrogen), giving a polyhistidine-tagged fusion protein expressed in *Escherichia coli*, BL21(DE3)LysS (Novagen). Oligonucieotide-directed mutagenesis at the codon for Ser 65 was performed[9] on the same construct using the Muta-Gene Phagemid *in vitro* kit version 2 (Bio-Rad). All fusion proteins were purified on nickel-chelate columns (Qiagen). Spectra were obtained from equal concentrations of GFPs controlled by densitometry of denaturing gels stained with Coomassie blue. Excitation spectra were obtained collecting emission at the respective peak wavelengths (508, 503, 507, and 511 nm for wild-type, S65A, S65C and S65T) and were corrected by a quantum counter; emission spectra were likewise measured at the respective excitation peaks (475, 471, 479, and 489 nm) and were corrected using factors from the fluorometer manufacturer. The amplitude of the 475 nm excitation peak of wild-type GFP has been defined as 1.0. The sixfold greater peak amplitude of S65T arises from a 5.5-fold higher extinction coefficient (39,200 $M^{-1}cm^{-1}$ at 490 nm for S65T compared with 21,000 and 7,150 $M^{-1}cm^{-1}$ at 395 and 475-nm for wild-type), similar fluorescence quantum yield (0.68 versus 0.77), and slightly narrower emission spectrum. The extinction coefficients and quantum yields reported here for recombinant wild-type protein fused to a polyhistidine tag are in good agreement with literature[6] values for GFP extracted from *Aequorea* when corrected for the revised molecular mass[2]. Recombinant expression and polyhistidine tagging are known[1,10,11] not to affect the fluorescence spectra of wild-type GFP. *b*, Rates of autoxidative fluorophore generation in wild-type (■) and S65T (▲) GFP, measured by development of fluorescence after admission of air to *E. coli* cultures anaerobically grown in GasPak pouches (Becton-Dickinson) for 3 days. Air was readmitted while transferring the cells to phosphate-buffered saline containing 8 mM NaN_3 as a metabolic inhibitor. The time course of subsequent fluorescence development measured the final oxidation step in the protein's self-modification to generate its internal fluorophore[8]. Data from two independent runs normalized by their respective asymptotic fluorescence values were pooled for each protein. The smooth curves are exponential curve fits consistent with pseudo-first-order kinetics, with time constants of 2.0 and 0.45 h for wild-type and S65T, respectively. Previously reported time constants for wild-type GFP autoxidation[8] were rather longer, probably because the protein was held in bacteria for longer periods of anaerobic growth, which seems to slow subsequent oxidation.

Kinetics of protein folding

SIR — Sali *et al.*[1] have attempted to resolve the "Levinthal paradox" of how proteins find their unique native conformations so fast. Although we agree with some of their points, we question others.

First, a model can bear on the Levinthal paradox only if the folding kinetics are run at a temperature low enough for the native state to be more stable than the denatured states. But Sali *et al.* are not studying native conditions: their molecules are mostly denatured. The temperatures they use are so high that equilibrium populations of the native states of many of their "folding sequences" are only 1–5% (ref. 2), and none exceeds 40% (ref. 1). Other model studies[3] show that native states can be accessed quickly in certain ranges of denaturing temperatures, but most of the chains will not stay there. If Sali *et al.* could not find native states under folding conditions, they have not completely addressed the Levinthal paradox.

Second, Sali *et al.* state that the "neces-